Microstructural and physiological responses to cadmium stress under different nitrogen levels in Populus cathayana females and males

Liu, Miao

2020-01

Liu , M , Bi , J , Liu , X , Kang , J , Korpelainen , H , Niinemets , U & Li , C 2020 , ' Microstructural and physiological responses to cadmium stress under different nitrogen levels in Populus cathayana females and males ' , Tree Physiology , vol. 40 , no. 1 , pp. 30-45 . https://doi.org/10.1093/treephys/tpz115

http://hdl.handle.net/10138/323968
https://doi.org/10.1093/treephys/tpz115

unspecified
acceptedVersion

The revision of ***TP-2019-286***

Microstructural and physiological responses to cadmium stress under different nitrogen levels in *Populus cathayana* females and males

Miao Liu [1], Jingwen Bi [1], Xiucheng Liu [1], Jieyu Kang [1],

Helena Korpelainen [2], Ülo Niinemets [3, 4, 5], Chunyang Li [1, *]

[1] College of Life and Environmental Sciences, Hangzhou Normal University, Hangzhou 310036, China

[2] Department of Agricultural Sciences, Viikki Plant Science Centre, University of Helsinki, P.O. Box 27, FI-00014, Finland

[3] Institute of Agricultural and Environmental Sciences, Estonian University of Life Sciences, Kreutzwaldi 1, 51006 Tartu, Estonia

[4] Estonian Academy of Sciences, Kohtu 6, 10130 Tallinn, Estonia

[5] School of Forestry and Bio-Technology, Zhejiang Agriculture & Forestry University, Hangzhou, Zhejiang, 311300, China

* Corresponding author: Chunyang Li, E-mail address: licy@hznu.edu.cn

Head title: Sexual differences in responses to Cd and N deficiency in poplar

Abstract Although an increasing attention has been paid on the relationships between heavy metal and nitrogen availability, the mechanism underlying adaptation to Cd stress in dioecious plants has been largely overlooked. This study examined Cd accumulation, translocation and allocation among tissues and cellular compartments in *Populus cathayana* females and males. Both leaf Cd accumulation and root-to-shoot Cd translocation were significantly greater in females than in males under a normal N supply, but they were reduced in females and enhanced in males under N deficiency. The genes related to Cd uptake and translocation, *HMA2*, *YSL2* and *ZIP2*, were strongly induced by Cd stress in female roots and in males under a normal N supply. Cd largely accumulated in the leaf blades of females and in the leaf veins of males under a normal N supply, while the contrary was true under N deficiency. Furthermore, Cd was mainly distributed in the leaf epidermis and spongy tissues of males, and in the leaf palisade tissues of females. N deficiency increased Cd allocation to the spongy tissues of female leaves and to the palisade tissues of males. In roots, Cd was preferentially distributed to the epidermis and cortices in both sexes, and also to the vascular tissues of females under a normal N supply but not under N deficiency. These results suggested that males possess better Cd tolerance compared to females, even under N deficiency, which is associated with their reduced root-to-shoot Cd translocation, specific Cd distribution in organic and/or cellular compartments, and enhanced antioxidation and ion homeostasis. Our study also provides new insights into engineering woody plants for phytoremediation.

***Keywords*:** dioecy, sexual differences, Cd distribution, nitrogen level, sequestration.

Introduction

Cadmium (Cd) is a nonessential and highly toxic element for plants. Cd is not only harmful to plant growth and metabolism but it also threatens human health, as large amounts of Cd may enter the food chain (Godt et al. 2006; Li et al, 2018). Phytoremediation by plants has been proposed to be an effective biotechnological strategy to remediate Cd-contaminated soils (Castagna et al. 2013, Li et al. 2018). Plants have evolved a series of strategies for Cd detoxification and tolerance. Cd could be sequestrated into cell walls and/or vacuoles, and it could induce antioxidant synthesis to alleviate oxidative stress (Peng et al. 2017, Zhang et al. 2018). Different plant species showhave different tolerances to Cd and employ different mechanisms to reduce Cd toxicity, but there can be different detoxification mechanism engaged even by the same species among different genotypes (Meyer et al. 2015, 2016).

Poplars have been suggested as promising candidates for remediating heavy metal-polluted soils due to their high growth rates and low impact on the food chain (Iori et al. 2016). Thus, both from the wood production and phytoremediation point of view it is important to improve poplars' growth and tolerance under Cd stress. The use of nitrogen fertilizers has been recently suggested to be one of the most important practices to alleviate Cd toxicity in plants (Chen et al., 2011; Liu et al., 2017), while Cd toxicity

affects nitrogen absorption and metabolism (Erdal & Turk, 2016). Cd inhibits NO_3^- uptake and impairs nitrate homeostasis, resulting in a decrease in nitrate transport from roots to shoots (Mao et al. 2014). Some enzymes related to nitrogen metabolism, such as nitrate reductase, glutathione synthase and glutamate synthetase, are also affected by Cd stress (Sharma et al. 2010, Erdal & Turk, 2016). In turn, some nitrogen metabolites, such as proline, glutathione (GSH) and phytochelatins (PCs), facilitate Cd detoxification in plants (Sharma & Dietz, 2006). Therefore, changes in the nitrogen status of plants may affect the stress caused by Cd.

Previous studies have suggested that responses of nitrogen metabolism to Cd stress vary among plant species, even among genotypes within the same species (Liao et al. 2019), as observed, e.g., in *Medicago sativa* (Yang et al. 2019). Furthermore, recent studies have shown that dioecious plants, e.g., *Populus* species, display sexual differences in defense responses to abiotic stress, including Cd toxicity, males usually displaying a better tolerance compared to females (Li et al. 2016, Chen et al. 2017), but the mechanisms causing sexual differences in Cd tolerance are poorly known. We have previously found that *P. yunnanensis* females are more sensitive to Cd stress than males, but N deposition could mitigate Cd toxicity and decrease sexual differences (Chen et al., 2011). However, sex-specific responses to combinations of Cd and N availability and underlying mechanisms have not been elucidated in *P. cathayana*. Therefore, we investigated physiological and molecular mechanisms related to nitrogen status and cadmium toxicity in *P. cathayana* males and females in order to reveal potential sex-

specific response patterns and to test the efficacy of enhanced N availability to alleviate Cd stress.

Materials and Methods

Plant material and growth conditions

Cuttings of *P. cathayana* females and males were collected from 60 different trees sampled in 15 populations, containing 30 females and 30 males, in the riparian and valley flat habitats of the Qinghai Province, China. Annual temperature, mean annual rainfall and annual solar radiation in the area are 6.9 °C (maximum 38 °C, minimum -20 °C), 335 mm and 4500 MJ m^{-2}, respectively (Zhao et al., 2009). Cuttings were rooted as described by Chen et al. (2015). The experimental design was with three factors (sex, N and Cd), i.e. two sexes (females, males), two Cd regimes (-Cd, +Cd) and two N levels (N deficiency, sufficient N). The seedlings were grown in a greenhouse at the Hangzhou Normal University. After one month, uniform cuttings were chosen and transplanted into plastic pots with a 10 kg mixture of sand, vermiculite and perlite (1:1:1). Every three days, 100 ml of nutrient solution and 100 ml of sterile distilled water were used for irrigation. The composition of the nutrient solution was as follows (μM): 500 μM KCl, 900 μM $CaCl_2$, 300 μM $MgSO_4$, 0.1 μM $CuSO_4$, 0.5 μM $MnSO_4$, 600 μM KH_2PO_4, 42 μM K_2HPO_4, 2000 μM NH_4NO_3, 25 μM Fe-EDTA, 10 μM H_3BO_3, 0.5 μM $ZnSO_4$, and 0.1 μM $(NH_4)_6Mo_7O_{24}$. The pH of the solution was adjusted to 6.0 using HCl. After

111 the seedlings had been growing in sandy pots for 30 d, uniform cuttings were subjected
112 to Cd and N treatments for 120 d. In the N treatment, the seedlings were irrigated with
113 a complete 2000 μM NH_4NO_3 (+N+Cd) or NH_4NO_3-free nutrient solution (-N+Cd),
114 and the final NH_4NO_3 level reached 200 mg N kg^{-1} soil. In the Cd treatment, $CdCl_2 \cdot$
115 2.5 H_2O of 100 μM was applied to the sandy pots every day during the first 40 d, and
116 the final Cd level reached 50 mg $CdCl_2 \cdot 2.5\ H_2O$ kg^{-1} dry soil.
117
118 *Growth measurements*
119
120 The plants were collected after the end of the experiments. Samples of roots, leaves and
121 stems were first oven-dried at 105 °C for 1 h and then dried at 70 °C until a constant
122 mass was reached, after which the dry mass (DM) was estimated.
123
124 *Gas exchange measurements and estimation of photosynthetic pigments*
125
126 The fourth fully expanded leaves were chosen to measure the net photosynthesis rate,
127 stomatal conductance and transpiration rate using the Ll-6400 photosynthesis
128 measuring system (Li-Cor, Inc., Lincoln, NE, USA) at 08:00-11:30 h. The measuring
129 conditions were as follows: 1500 μmol $m^{-2}s^{-1}$ photosynthetic photon flux density, 25 °C
130 leaf temperature, 70% air humidity and 400 μmol mol^{-1} ambient CO_2 concentration. In
131 addition, the leaves were extracted in 80% cooled acetone (v/v) in the dark until the
132 leaves changed their color to white. Chlorophyll and carotenoid concentrations were

measured from measurements of solution absorbances at 470, 646 and 663 nm, and calculated according to Chen et al. (2011).

Determination of reactive oxygen species and enzyme activities

The reactive oxygen species, malondialdehyde (MDA) and enzyme activities were measured according to the method by Chen et al. (2011). For H_2O_2, c. 0.2 g of leaves and roots were ground with liquid nitrogen and then with 5% trichloroacetic acid, followed by Chen et al. (2011). Briefly, 0.2 ml of clear supernatant was mixed with 1 ml 20% $TiCl_4$ (v/v, dissolved in HCl) and 0.2 ml ammonia, and then centrifugated at 5000 *g* for 10 min. The precipitation was dissolved in 1.5 M H_2SO_4, and measured at 410 nm. For O_2^- determination, the leaves and roots were finely ground with the extraction mixture (50 mM Na_2HPO_4-NaH_2PO_4, pH 7.8) and then centrifugated at 12000 *g* for 10 min. A volume of 0.5 ml of supernatant was mixed with 0.1 ml hydroxylamine hydrochloride (10 mM). The reaction was conducted at 25 °C for 30 min. O_2^- levels were measured colorimetrically at 540 nm after adding 1 ml of 0.2% N-(1-naphthyl)-ethylene diamine and 1 ml of 1% sulfanilamide. For MDA, the leaves and roots were ground with 10% trichloroacetic acid and centrifuged at 12000 *g* for 10 min. Then, 0.5 ml of clear supernatant was let to react with 2 ml thiobarbituric acid (0.6%) in a boiling water bath for 15 min. MDA was measured colorimetrically at 450, 320 and 600 nm, and calculated as follows: C (nM) = 6.45 (OD_{532}-OD_{600})-0.56×OD_{450}.

The activities of peroxidase (POD), superoxide dismutase (SOD), glutathione (GR) and

155 catalase (CAT) were measured as described by Chen et al. (2011). Proteins were
156 measured using the Bradford method.
157
158 *Determination of Cd and nutrient elements*
159
160 Dried leaves and roots were finely ground and dissolved in 3:1 (v/v) of HNO_3 and
161 $HClO_4$. Total Cd and nutrient elements were measured with ICP-MS (inductively
162 coupled plasma mass spectrometer Agilent 7500a, Agilent Technologies). The
163 translocation factor (T_f) was defined as the ability for root-to-shoot Cd translocation
164 and calculated as the ratio of Cd concentration in shoots to roots (Shi et al. 2010).
165
166 *Microscopic imaging of Cd, P and S localization in roots, leaf blades and veins*
167
168 Leaves and roots were washed carefully with deionized H_2O. Subsequently, the samples
169 were cut into sections and dried with a vacuum freeze dryer for 100 h. The sample
170 surfaces were gold-plated with vacuum sputtering. Photographs were taken under a
171 scanning electron microscope (Zeiss Sigma 500, German) at 3 kV. The line scan on the
172 sample surface was conducted with an energy-dispersive x-ray (EDX) (EDAX
173 ELEMENT, America) at 10 kV voltage. The spectra of Cd, P and S on the surface was
174 analyzed with the SuperQuant program (EDAX).
175
176 *Quantitative PCR analyses of gene expression related to Cd uptake and transport*

177

178 Approximately 0.1 g of roots and leaves was finely ground with liquid nitrogen. Total
179 RNA was isolated using a RNA extraction kit (TaKaRa MiniBEST Plant RNA
180 Extraction Kit, TaKaRa, Otsu, Japan). The first cDNA strand was synthesized using
181 PrimeScript reverse transcription (RT) reagent kits (Takara) according to instructions.
182 Quantitative RT polymerase chain reactions (qRT-PCR) were conducted with One Step
183 TB Green™ PrimeScript™ RT-PCR kits in a 25 μl reaction system with pairs of
184 specific primers (He et al. 2013) (Table S1).

185

186 Heavy metal ATPase 2 and 4 (HMA2 and HMA4) proteins facilitate the root-to-shoot
187 translocation of Cd (Li et al. 2018). Metallothionein-like protein (MTP1) and yellow
188 stripe-like protein (YSL2) are responsive for Cd transportation into the vacuoles
189 (Ricachenevsky et al. 2013). The zinc transporter 2 and 6.2 (ZIP2 and ZIP6.2) regulate
190 the Cd translocation into the cell cytosol of roots (Ma et al. 2014). These genes were
191 analyzed in this study. The primers were similar as those in He et al. (2015). *TUB4.1*
192 was used as housekeeping genes. The relative expression of specific genes was
193 calculated according to Liu et al. (2017).

194

195 *Analysis of Fourier transform infrared spectroscopy (FTIR)*

196

197 The leaves and roots were washed carefully with deionized H_2O. Subsequently, the
198 samples were dried with a vacuum freeze dryer for 100 h. The freeze-dried powder of

leaves and roots was pressed against the diamond crystal of an attenuated total reflectance device and the infra-red spectra were determined with FTIR spectrometer Nicolet iS5. The scanning range was 400-4000 cm^{-1} wavenumber.

As shown in Table S2, the differential spectral peaks are at 1651 cm^{-1} for C-N vibration from protein, at 1419 cm^{-1} for vibration of COO^- from pectin, at 1317 cm^{-1} for C-O vibration from cellulose, at 1151 cm^{-1} for vibration of C-C and C-O stretch from carbohydrates (such as soluble sugar, cellulose and hemicellulose), at 1235 cm^{-1} for C=O vibration from xylans and lignin, at 1071 cm^{-1} for C-O from cellulose and hemicellulose, at 1743 cm^{-1} for vibration of C=O from esterified pectin, and at 1111 cm^{-1} for C-C or C-O vibration from pectin.

Statistical analysis

Differences among means within treatments were separated by Duncan's test using the SPSS software (version 22.0) with three-way analyses when $P < 0.05$. Data were checked for the normality before analyses. The principal component analysis (PCA) was computed by the command procomp () in R (http://www.R-project.org/) according to Luo et al. (2019).

Results

Sexual differences in leaf gas exchange characteristics, pigments and biomass

Cd stress, N deficiency and the combined stress reduced A in both sexes, especially in females (Table 1). The stomatal conductance (g_s) was not affected by stress in females, but it was significantly reduced in males under the combined treatment (Table 1). All stresses increased the intercellular CO_2 concentration (C_i) in both females and males (Table 1). The chlorophyll concentrations of a, b, a+b, and carotenoids were reduced in females, while no change was found in males under any stress. Additionally, N deficiency did not affect these chlorophylls relative to a normal N supply in either sex exposed to Cd stress.

In females, the dry mass of leaves, stems and roots, and total plant dry mass decreased under nitrogen deficiency and Cd stress, and more seriously under the combined treatment (Table 2). In males, these values decreased under nitrogen deficiency and combined stress, but Cd stress did not affect leaf dry mass when compared to Cd-free conditions. Under control conditions (high N, no Cd), females showed a higher leaf and root dry mass, and total biomass, but a lower stem dry mass when compared to males. In addition, N deficiency significantly increased the ratio of root to shoot irrespective of the Cd treatment in both sexes.

Sexual differences in oxidative stress and antioxidants

243 N deficiency, Cd stress and the combined treatment increased H_2O_2 and O_2^- of leaves,
244 and MDA, H_2O_2 and O_2^- of roots in females compared with control plants. Males
245 showed higher H_2O_2 in roots and leaves under all stresses when compared to controls.
246 O_2^- in male roots and leaves was not affected by Cd stress, but its concentration
247 increased in male roots under N deficiency, and in leaves under N deficiency and the
248 combined stress. Furthermore, females showed higher MDA in roots and O_2^- in leaves
249 under all stress conditions compared to males. There was no significant difference
250 between sexes in leaf MDA under N deficiency, in leaf H_2O_2 under Cd stress, or in root
251 H_2O_2 under the combined treatment. In contrast, males had higher H_2O_2 in leaves under
252 the combined treatment and in roots under N deficiency alone.
253
254 In males, Cd stress, N deficiency and the combined treatment significantly increased
255 SOD in roots and leaves, as well as CAT in roots, but it did not affect POD and GR in
256 roots (Fig. 2). In females, POD and GR of roots were reduced under all stresses, while
257 CAT of roots and leaves, as well as POD and GR of leaves increased under Cd stress.
258 The combined stress increased CAT and SOD in leaves and SOD in roots in females.
259 When compared to females, males had higher POD in leaves, CAT and POD in roots,
260 and SOD in roots and leaves under the combined treatment (Fig. 2). However, there
261 was no significant difference between sexes in GR of roots under N deficiency and Cd
262 stress, and in POD of roots and leaves under the combined stress. CAT in leaves under
263 N deficiency and the combined stress, and SOD in roots under the combined stress were
264 higher in females than in males.

265

266 *Sexual differences in Cd accumulation*

267

268 Elevated Cd exposure strongly increased Cd accumulation in leaves, roots, stem wood
269 and bark in both sexes (Fig. 3). Males had higher Cd levels in roots, but lower Cd in
270 leaves compared to females under a normal N supply. Cd levels in barks and stem
271 woods were not different between females and males under a normal N supply and Cd
272 stress (+N+Cd). When compared to a normal N supply, N deficiency combined with
273 Cd stress strongly promoted Cd accumulation in leaves, roots and woods of males,
274 while females had lower Cd levels in leaves, barks and woods. Cd in roots of females
275 and in barks of males showed no differences between N deficiency and a normal N
276 supply under Cd stress (Fig. 3). Additionally, males had higher Cd in leaves, roots,
277 woods and barks than females under the combined stress. The translocation factor T_f
278 was significantly higher in females than in males under Cd stress, but no significant
279 difference was detected under the combined treatment (Fig. S1).

280

281 *Sexual differences in Cd allocation within organs*

282

283 The energy-dispersive x-ray (EDX) and scanning electron microscope (TEM) were
284 used to explore Cd distribution in the cross-sections of leaf blades, leaf veins and roots.
285 Females had a stronger Cd signal in leaf vein cross-sections compared to males under
286 control conditions (Fig. 4). N deficiency reduced Cd allocation into vein cross-sections

in both sexes, but more strongly in males. Furthermore, in females, a large amount of Cd distributed into epidermal and cortical tissues, as well as into vascular tissues, especially into phloem under a normal N supply. In contrast, N deficiency increased Cd allocation to the leaf vein phloem, epidermis and cortices, especially to the upper epidermis of leaf veins in females (Fig. 4). In males, more Cd was allocated to the cortices of the abaxial veins of leaves, as well as to vascular tissues, especially in xylems. N deficiency combined with Cd stress induced considerable Cd allocation to the epidermis and cortices of the abaxial veins, as well as to xylems in males.

In leaf blade cross-sections, the Cd signal intensity was higher in females than in males under a normal N supply (Fig. 5). N deficiency increased the intensity of Cd signals throughout the male leaf blades, but reduced them in females. Specifically, more Cd distributed into the mesophyll of females, while males had strong Cd signals in the upper epidermis and mesophyll under a normal N supply. N deficiency increased the Cd distribution in epidermal tissues in females, especially in the upper epidermis (Fig. 5). In contrast, more Cd was allocated to the mesophylls and lower epidermal tissues of male leaf blades under the combined treatment. Furthermore, Cd signals largely distributed in female mesophylls, especially in the palisade tissues, while males had high Cd in the spongy tissues under a normal N supply (Fig. 5). N deficiency largely increased the proportion of Cd allocation to the spongy tissues of leaf blades in both sexes when compared to normal N supply conditions.

309 In roots, males had slightly higher Cd signals throughout the cross-sections when
310 compared to females under a normal N supply (Fig. 6). N deficiency increased Cd
311 accumulation in the cross-sections of males, but reduced Cd signals in females. Under
312 a normal N supply, Cd signals were strongest in the epidermal, cortical and vascular
313 tissues of females, while males had more Cd in epidermal and cortical tissues. N
314 deficiency induced more Cd allocation to epidermal and cortical tissues, and less to
315 vascular tissue in females (Fig. 6). However, more Cd signals were detected in the
316 epidermal, cortical and vascular tissues of males under the combined treatment.
317
318 *Sexual differences in S and P allocation among tissues*
319
320 The allocation of P and S were also studied in the cross-sections of roots, leaf veins and
321 blades by the application of EDX analysis and SEX imaging. In leaf blades, females
322 had a higher P to S ratio, while the contrary was true for males under a normal N supply
323 (Fig. S2). N deficiency increased the proportion of S in both sexes. Cd stress increased
324 S in leaf blade cross-sections relative to P in both sexes, especially in females treated
325 without a N supply and in males treated with a normal N supply (Fig. 7).
326
327 In leaf vein cross-sections, the ratio of S to P signal was higher in epidermal and cortical
328 tissues of females when compared to males but not in vascular tissues (Fig. S2). Males
329 had stronger S signals than P signals under a normal N supply, and S and P were
330 uniformly distributed throughout the cross-sections of leaf veins under both N levels

(N deficiency, normal N supply). In females, N deficiency induced P allocation to vascular tissues, but the proportions of P and S were similar in epidermal and cortical tissues. Cd stress significantly increased S throughout leaf vein cross-sections in both sexes, especially in males (Fig. 7). Moreover, P and S were mainly distributed in the vascular tissues of males under N deficiency. The proportion of S relative to P in roots was highest in N-sufficient females and N-deficient males (Fig. S2). Cd stress increased P in female roots under a normal N supply and P of males under N deficiency relative to Cd-free controls (Fig. 7).

FTIR spectra of roots and leaves

To investigate the effect of Cd on the chemical fingerprint of the molecular composition of the cell wall, we measured the absorption spectra peaks of leaves and roots. In this study, PCA analysis was performed to study the original absorbance data. In leaves, PC1 and PC2 accounted for 92% and 2% of the variation, respectively (Fig. 8; Table S3). Peaks at 1651, 1419, 1317 cm^{-1} were the most vital contributors to PC1, whereas peaks 1111 and 1541 cm^{-1} were the key factors contributing to PC2. In roots, PC1 and PC2 accounted for 95% and 2% of the variation, respectively. In the PCA plot of roots, peaks 1419, 1317, 1111 and 1157 cm^{-1} were key factors contributing to PC1, whereas peaks at 3360 cm^{-1} and 2915 cm^{-1} were main contributors to PC2 (Fig. 8; Table S4). The PCA results indicated that males showed more significant changes in the chemical composition of leaves and roots compared with females under both N supply levels, but

353 especially under a normal N supply, reflecting the absorption by the groups related to
354 lignin, cellulose, hemicellulose and pectin in roots and leaves.
355
356 *PCA of physiological responses*
357
358 To uncover the main factors participating in the adaptive responses of females and
359 males to Cd stress and N deficiency, PCA was performed using traits related to
360 photosynthesis, growth, element concentrations, oxidative stress and antioxidative
361 capacity (Fig. 9; Table S5). PC1 and PC2 accounted for 40% and 19% of the variation,
362 respectively. Shoot dry mass, stem mass, net photosynthesis rate and leaf H_2O_2 levels
363 were key factors contributing to PC1, while root H_2O_2 levels, g_s and transpiration rates
364 were the three most important factors contributing to PC2. The PCA separated females
365 from males in responses to Cd stress and N deficiency.
366
367 *Transcript levels of genes involved in Cd translocation and uptake*
368
369 We analyzed the genes related to Cd translocation and tolerance in roots. Cd induced
370 the expression of *HMA2* and *HMA4* genes in female roots, whereas in male roots Cd
371 stress downregulated *HMA2*i but did not affect *HMA4* expression compared to the
372 controls (Fig. 10). N deficiency did not affect the expressions of *HMA2* and *HMA4* in
373 female roots, but it upregulared the expression of *HMA2* under Cd stress compared to
374 controls. Cd stress induced *MTP1* gene expression in female roots, especially under N

375 deficiency, while the transcription of *MTP1* in male roots was strongly induced by Cd
376 stress but down-regulated under the combined stress. The expression of *YSL2* in roots
377 was induced in females by Cd stress but inhibited in males, while it was upregulated by
378 the combined stress in both sexes. The transcription levels of *ZIP2* and *ZIP6.2* genes in
379 male roots and *ZIP2* of female roots were upregulated by Cd stress irrespective of the
380 N status, while the expression of *ZIP6.2*gene in female roots was not affected by Cd
381 stress but downregulated under the combined stress.
382
383 **Discussion**
384
385 *Sexually different physiological tolerance to Cd stress and N deficiency*
386
387 Cadmium interferes with plant growth and metabolism, but the toxic effects of Cd differ
388 among plant species (Baliardini et al. 2015, He et al. 2015). In this study, Cd stress
389 significantly reduced A and leaf dry mass in females but not in males under a normal N
390 supply (Tables 1, 2). This result is consistent with previous studies (Chen et al. 2011;
391 Chen et al. 2016). Noticeably, the damage on photosynthesis and biomass accumulation
392 was smaller in males than in females under the combined stress (Table 2). Interestingly,
393 we also found that males showed no significant symptoms of Cd toxicity irrespective
394 of the N supply, while clear Cd toxicity symptoms were found in the abaxial leaves of
395 females under a normal N supply but not under N deficiency (Fig. S3). It could be
396 inferred that males have a far stronger Cd tolerance compared to females, especially

under a normal N supply (Fig. S3). Yet, Cd toxicity symptoms of females did not differ much between N deficiency and a normal N supply, which could be explained by the reduced Cd translocation to leaves (Fig. S1). Our results appeared different from those of Chen et al (2011), who proposed that N deposition decreases differences in Cd sensitivity in *Populus yunnanensis* females and males. However, the N supply level and species were different in the study by Chen et al (2011). They used normal and higher N applications, while we used N deficiency and a normal N supply. It seems that the N availability (N deficiency, normal N and high N) differently affects the responses of females and males to Cd stress.

Cd accumulation in leaves disrupts photosynthesis (Fei et al. 2018). Thus, the inhibition of Cd translocation to the shoots is probably an effective approach to enhance Cd tolerance (Daud et al. 2015, Fei et al. 2018). Cd uptake, root-to-shoot translocation and accumulation in leaves were largely induced under a normal N supply, while N deficiency reduced Cd accumulation in the leaves of females (Fig. 3). This is in accordance with previous studies (Chang et al., 2013, Hu et al., 2013). The lower Cd accumulation in female leaves under N deficiency is probably a self-protecting strategy to cope with Cd toxicity, as described by Zhang et al (2019), who suggested that lower Cd accumulation in *Populus* leaves might be a self-protecting strategy to prevent severe oxidative damage due to a decreased stress tolerance under N deficiency. Cheng et al (2017) also suggested that ammonium-based fertilizers enhance Cd accumulation in *Carpobrotus rossii*. However, this is not the case in *P. cathayana* males, since more Cd

419 accumulated in leaves under N deficiency than under a normal N supply (Fig. 3).
420 Similarly, Konotop et al (2012) suggested that a nitrogen application decreases Cd
421 uptake and improves Cd tolerance in soybean seedlings. Perilli et al (2010) have also
422 proposed that cadmium concentrations of wheat are influenced by the nitrogen level,
423 seedling age and soil type. The greater Cd accumulation in male leaves under N
424 deficiency is probably correlated with N sensitivity, since males have a lower resource
425 consumption and stronger tolerance to N deficiency compared to females. Moreover,
426 the capacity for antioxidation defense was significantly elevated by N deficiency in
427 males (Fig. 2). Evidently, males and females employ different mechanisms to cope with
428 Cd toxicity, especially under N deficiency.
429
430 *Changed nutrient allocation and oxidative-antioxidation homeostasis in females and*
431 *males under N deficiency highlight the physiological regulation mechanism of Cd*
432
433 *Populus* species are characterized by dioecy, which is usually associated with sexual
434 dimorphism. Females generally allocate more resources to reproduction and males
435 often increase investment in defense (Juvany & Munné-Bosch, 2015). Sulfur
436 compounds, such as GSH, PCs and metallothionein, act as antioxidants or chelators
437 involved in plant tolerance to heavy metals (Cobbett & Goldsbrough 2002, Li et al.
438 2019). Therefore, P and S allocation to roots, leaf blades and veins exposed to Cd stress
439 were analyzed in this study (Fig. 7). Females had higher proportions of P relative to S
440 in leaves under control conditions, especially in leaf blades, which is consistent with

441 their higher reproductive investment (Graff et al. 2013). In contrast, the proportion of
442 S relative to P was higher in males under control conditions, which is consistent with
443 their stronger tolerance to stress (He et al. 2013). S and P allocations were closely
444 correlated with Cd throughout cross-sections of leaf blades and veins in both sexes,
445 especially in males under a normal N supply and in females under N deficiency (Figs.
446 4-7). Cd detoxification by S has been previously reported (Sarwar et al. 2010, Chen et
447 al. 2015). In addition, He et al (2015) found in poplars that S was significantly
448 correlated with Cd and it increased GSH synthesis through the overexpression of
449 bacterial γ-glutamylcysteine synthetase facilitated by Cd detoxification and enhanced
450 Cd tolerance. Hence, the stronger tolerance of males is probably attributable to the high
451 proportion of S in males under Cd stress. It should be noted that N deficiency increased
452 the proportion of P in males under Cd stress. P is involved in PC biosynthesis, Cd is
453 transported into vacuoles by Cd/PC complexes, and Cd is sequestrated into cell walls
454 through an association with phosphates (Parrotta et al. 2015). Hence, the increase in P
455 induced by N deficiency probably plays an important role in the Cd tolerance of males.
456
457 Cd toxicity is often accompanied with a ROS burst, which causes the disruption of
458 redox homeostasis, followed by oxidative damage on plant cells (Rui et al. 2016, Gupta
459 et al. 2017). The present study found that Cd induces oxidative damage more seriously
460 in females than in males, especially by O_2^- in leaves and by MDA in roots (Fig. 1). ROS
461 act to stimulate an early defective response (Liu et al. 2018a, b), and elevated H_2O_2
462 levels detected in male and female leaves and roots are early signals of adaptive

responses to stress, including the induction of antioxidants. Antioxidants, such as GR, SOD, POD and CAT, are regarded as main enzymatic antioxidants scavenging the detrimental effects of ROS in plants (Schutzendubel et al. 2001). Cd stress reduced CAT, POD and GR activities in female leaves and roots but had little effect on males compared to controls (Fig. 2). Furthermore, although N deficiency promoted Cd accumulation in male leaves but reduced that in females, females were still more sensitive to Cd stress compared to males.

Sexually different Cd sequestration and accumulation among tissues under N deficiency highlight the mechanism of Cd tolerance

It is worth noting that N deficiency reduced Cd uptake and/or translocation in females but increased those in males under Cd stress. The extensive Cd accumulation in shoots without toxicity symptoms is similar as what happens in hyperaccumulator plants, which are characterized by a high capacity of root-to-shoot translocation (Lu et al. 2013). We found that genes related to Cd uptake and translocation, such as *HMA2* and *HMA4*, *YSL2* and *ZIP2*, were strongly induced by Cd stress in female roots under a normal N supply (Fig. 10). In male roots, the expression of *HMA2*, *YSL2* and *ZIP6* genes were significantly induced by N deficiency. Additionally, strong sequestration into the cell walls of male roots under a normal N supply reduced Cd accumulation in shoots (Fig. 8).

485 Males showed a better Cd tolerance when compared to females under both N supply
486 levels, although N deficiency promoted Cd uptake and/or translocation from roots to
487 shoots in males (Fig. 3, Fig. S1). This is consistent with observations on the
488 hyperaccumuating ecotype of *Sedum alfredii Hance*, in which Cd accumulates in shoots
489 without toxicity symptoms (Tian et al. 2017). Successful Cd detoxification probably
490 requires effective sequestration of Cd into organs (Lu et al. 2013), and effective Cd
491 sequestration among tissues and cellular compartments, as observed in *P. cathayana*
492 males, is probably accomplished as a permanent Cd storage (Tian et al. 2009). We found
493 that Cd largely accumulates in male bark, especially under N deficiency (Fig. 3), which
494 is consistent with previous investigations (He et al. 2013). Epidermal Cd increased in
495 leaf veins and blades, primarily in the upper leaf epidermis and spongy tissues of males,
496 especially under N deficiency, perhaps protecting leaf mesophylls and guard cells
497 against Cd toxicity (Figs. 4-5). Furthermore, successful Cd detoxification probably
498 need effective sequestration in appropriate cellular compartments to accomplish the
499 status of permanent storage (Tian et al. 2013). The cell walls and vacuoles are suggested
500 to effectively sequestrate Cd and reduce cytosolic Cd levels in plants (Peng et al. 2017,
501 Zhang et al. 2018). In this study, Cd stress induced the expression of *HMA2*, *HMA4* and
502 *MTP1* genes in male leaves, especially the expression of *MTP1* under N deficiency (Fig.
503 S4). Additionally, the higher ratio between males and females at 1419, 1317, 1111 and
504 1157 cm^{-1} of FTIR suggested that higher pectin and lignin levels probably contributed
505 to Cd detoxification in males.
506

Conclusions

The present study suggests that one of the primary factors responsible for a greater Cd allocation to leaves and sensitivity in females is ineffective Cd sequestration in organs and/or cellular compartments (Fig. 11). Moreover, the more extensive root-to-shoot translocation of Cd and the weaker Cd detoxification in females also led to their greater sensitivity to Cd toxicity, irrespectively of the N supply. Although N deficiency reduced the Cd root-to-shoot translocation in females and elevated that in males, males had a better Cd tolerance compared to females under Cd stress. It follows that it may be important to modify artificially the soil N status depending on the Cd tolerance of *P. cathayana* females and males. In all, our investigation provides new insights into efforts aiming to engineer woody plants for phytoremediation.

Acknowledgements This work was supported by the Natural Science Foundation of China (31800507) and the Talent Program of the Hangzhou Normal University (2016QDL020).

Author contributions Miao Liu had the main responsibility for data collection, analysis and writing, Jingwen Bi, Xiucheng Liu and Jieyu Kang contributed to data collection and analysis, Helena Korpelainen and Ülo Niinemets contributed to the interpretation of data and manuscript preparation, and Chunyang Li (the corresponding author) had the overall responsibility for experimental design and project management.

Conflict of interest The authors declare that they have no conflict of interest.

Supporting Information Additional supporting information and references can be found in the supplementary information.

References

Baliardini C, Meyer CL, Salis P, Saumitou-Laprade P, Verbruggen N (2015) CAX1 co-segregates with Cd tolerance in the metal hyperaccumulator *Arabidopsis halleri* and plays a role in limiting oxidative stress in Arabidopsis. Plant Physiol 169: 549-559.

Castagna A, Di Baccio D, Tognetti R, Ranieri A, Sebastiani L (2013) Differential ozone sensitivity interferes with cadmium stress in poplar clones. Biol Plantarum 57: 313-324.

Chang YS, Chang YJ, Lin CT, Lee MC, Wu CW, Lai YH (2013) Nitrogen fertilization promotes the phytoremediation of cadmium in *Pentas lanceolata*. Int Biodeter Biodegr 85: 709-714.

Chen J, Duan B, Xu G, Korpelainen H, Niinemets Ü, Li C (2016) Sexual competition affects biomass partitioning, carbon–nutrient balance, Cd allocation and ultrastructure of *Populus cathayana* females and males exposed to Cd stress. Tree Physiol 36: 1353-1368.

Chen J, Han Q, Duan B, Korpelainen H, Li C (2017) Sex-specific competition

551 differently regulates ecophysiological responses and phytoremediation of *Populus*
552 *cathayana* under Pb stress. Plant Soil 421: 203-218.
553 Chen L, Han Y, Jiang H, Korpelainen H, Li C (2011) Nitrogen nutrient status induces
554 sexual differences in responses to cadmium in *Populus yunnanensis*. J Exp Bot 62:
555 5037-5050.
556 Chen J, Yang LB, Gu J, Bai XY, Ren YB, Fan TT, Han Y, Jiang L, Xiao FM, Liu YS,
557 Cao SQ (2015) *MAN3* gene regulates cadmium tolerance through the glutathione-
558 dependent pathway in *Arabidopsis thaliana*. New Phytol 205: 570-582.
559 Cheng MM, Wang A, Tang CX 2017 Ammonium-based fertilizers enhance Cd
560 accumulation in *Carpobrotus rossii* grown in two soils differing in pH. Chemosphere
561 188: 689-696.
562 Cobbett C & Goldsbrough P (2002) Phytochelatins and metallothioneins: roles in heavy
563 metal detoxification and homeostasis. Ann Rev Plant Biol 53: 159-182.
564 Daud MK, Quiling H, Lei M, Ali B, Zhu SJ (2015) Ultrastructural, metabolic and
565 proteomic changes in leaves of upland cotton in response to cadmium stress.
566 Chemosphere 120: 309-320.
567 Erenoglu EB, Kutman UB, Ceylan Y, Yildiz B, Cakmak I (2011) Improved nitrogen
568 nutrition enhances root uptake, root-to-shoot translocation and remobilization of zinc
569 (65^{Zn}) in wheat. New Phytol 189(2): 438-448.
570 Erdal S, Turk H (2016) Cysteine-induced upregulation of nitrogen metabolism-related
571 genes and enzyme activities enhance tolerance of maize seedlings to cadmium stress.
572 Environ Exp Bot 132: 92-99.

573 Fei L, Xu P, Dong Q, Mo Q, Wang Z (2018) Young leaf protection from cadmium
574 accumulation and regulation of nitrilotriacetic acid in tall fescue (*Festuca*
575 *arundinacea*) and Kentucky bluegrass (*Poa pratensis*). Chemosphere 212: 124-132.
576 Graff P, Rositano F, Aguiar MR (2013) Changes in sex ratios of a dioecious grass with
577 grazing intensity: the interplay between gender traits, neighbor interactions and
578 spatial patterns. J Ecol 101: 1146-1157.
579 Godt J, Scheidig F, Grosse-Siestrup C, Esche V, Brandenburg P, Reich A, Groneberg
580 DA 2006 The toxicity of cadmium and resulting hazards for human health. Journal
581 of occupational medicine and toxicology 1(1): 22.
582 Gupta DK, Pena LB, Romero–Puertas MC, Hernández A, Inouhe M, Sandalio LM
583 (2017) NADPH oxidases differentially regulate ROS metabolism and nutrient uptake
584 under cadmium toxicity. Plant Cell Environ 40: 509-526.
585 He JL, Li H, Luo J, Ma CF, Li SJ, Qu L, Gai Y, Jiang XN, Janz D, Polle A, Tyree M
586 (2013) A transcriptomic network underlies microstructural and physiological
587 responses to cadmium in *Populus*× *canescens*. Plant Physiol162: 424-439.
588 He JL, Li H, Ma CF, Zhang YL, Polle A, Rennenberg H, Cheng XQ, Luo ZB (2015)
589 Overexpression of bacterial γ-glutamylcysteine synthetase mediates changes in
590 cadmium influx, allocation and detoxification in poplar. New Phytol 205: 240-254.
591 Hu P, Yin YG, Ishikawa S, Suzui N, Kawachi N, Fujimaki S, Igura M, Yuan C, Li HZ,
592 et al (2013) Nitrate facilitates cadmium uptake, transport and accumulation in the
593 hyperaccumulator *Sedum plumbizincicola*. Environ Sci Pollut R 20(9): 6306-6316.
594 Iori V, Gaudet M, Fabbrini F, Pietrini F, Beritognolo I, Zaina G, Mugnozza GS, Zacchini

595 M, Massacci A, Sabatti M (2016) Physiology and genetic architecture of traits
596 associated with cadmium tolerance and accumulation in *Populus nigra* L. Trees 30:
597 125-139.
598 Juvany M, Munné-Bosch S (2015) Sex-related differences in stress tolerance in
599 dioecious plants: a critical appraisal in a physiological context. J Exp Bot 66: 6083-
600 6092.
601 Konotop Y, Mezsaros P, Matusikova I, Batsmanova L, Taran N (2012) Application of
602 nitrogen nutrition for improving tolerance of soybean seedlings to cadmium. Environ
603 Exp Biol 10: 139-144.
604 Li NN, Xiao H, Sun JJ, Wang SF, Wang, JC, Chang P, Zhou XB, Lei B, Lu Kun, Luo F
605 et al (2018) Genome-wide analysis and expression profiling of the *HMA* gene family
606 in *Brassica napus* under cd stress. Plant Soil 426: 365-381.
607 Li Y, Duan B, Chen J, Korpelainen H, Niinemets Ü, Li C (2016) Males exhibit
608 competitive advantages over females of *Populus deltoides* under salinity stress. Tree
609 Physiol 36: 1573-1584.
610 Li Y, Liu K, Wang Y, Zhou Z, Chen C, Ye P, Yu F (2018) Improvement of cadmium
611 phytoremediation by *Centella asiatica* L. after soil inoculation with cadmium-
612 resistant Enterobacter sp. FM-1. Chemosphere 202: 280-288.
613 Li XZ, Yu H, Sun XW, Yang JT, Wang D,C Shen LF, Pan YS, Wu YC, Wang Q, Zhao
614 Y (2019) Effects of sulfur application on cadmium bioaccumulation in tobacco and
615 its possible mechanisms of rhizospheric microorganisms. J Hazard Mater 368: 308-
616 315.

617 Liu M, Liu XX, He XL, Liu LJ, Wu H, Tang CX, Zhang YS, Jin CW (2017) Ethylene
618 and nitric oxide interact to regulate the magnesium deficiency-induced root hair
619 development in Arabidopsis. New Phytol 213: 1242-1256.
620 Liu M, Bi JW, Jin CW (2018a) Developmental responses of root hairs to Mg deficiency.
621 Plant Signaling & Behavior 13: e1500068.
622 Liu M, Zhang HH, Fang XZ, Zhang Y, Jin CW (2018b) Auxin acts downstream of
623 ethylene and nitric oxide to regulate magnesium deficiency-induced root hair
624 development in *Arabidopsis thaliana*. Plant Cell Physiol 59: 1452-1465.
625 Liu M, Sun J, Li Y, Xiao Y (2017) Nitrogen fertilizer enhances growth and nutrient
626 uptake of *Medicago sativa* inoculated with *Glomus tortuosum* grown in Cd-
627 contaminated acidic soil. Chemosphere 167: 204-211.
628 Liao Q, Jian SF, Song HX, Guan CY, Lepo JE, Ismail AM, Zhang ZH (2019) Balance
629 between nitrogen use efficiency and cadmium tolerance in *Brassica napus* and
630 *Arabidopsis thaliana*. Plant Sci (online).
631 Lu L, Tian S, Zhang J, Yang X, Labavitch JM, Webb SM, Latimer M, Brown PH (2013)
632 Efficient xylem transport and phloem remobilization of Zn in the hyperaccumulator
633 plant species *Sedum alfredii*. New Phytol 198: 721-731.
634 Luo J, Zhou JJ, Masclaux-Daubresse C, Wang N, Wang H, Zheng B (2019)
635 Morphological and physiological responses to contrasting nitrogen regimes in
636 *Populus cathayana* is linked to resources allocation and carbon/nitrogen partition.
637 Environ Exp Bot 162: 247-255.
638 Ma YL, He JL, Ma, CF, Luo J, Li H, Liu TX, Polle A, Peng CH, LUO ZB (2014)

639 Ectomycorrhizas with *Paxillus involutus* enhance cadmium uptake and tolerance in
640 *Populus* × *canescens*. Plant, Cell Environ 37: 627-642.
641 Mao QQ, Guan MY, Lu KX, Du ST, Fan SK, Ye YQ, Lin XY, Jin CW (2014) Inhibition
642 of nitrate transporter 1.1-controlled nitrate uptake reduces cadmium uptake in
643 Arabidopsis. Plant Physiol 166: 934-944.
644 Meyer CL, Pauwels M, Briset L, Godé C, Salis P, Bourceaux A, Souleman D, Frérot H,
645 Verbruggen N (2016) Potential preadaptation to anthropogenic pollution: evidence
646 from a common quantitative trait locus for zinc and cadmium tolerance in
647 metallicolous and nonmetallicolous accessions of *Arabidopsis halleri*. New Phytol
648 212: 934-943.
649 Meyer CL, Juraniec M, Huguet S, Chaves-Rodriguez E, Salis P, Isaure MP,
650 Goormaghtigh, Verbruggen N (2015) Intraspecific variability of cadmium tolerance
651 and accumulation, and cadmium-induced cell wall modifications in the metal
652 hyperaccumulator *Arabidopsis halleri*. J Exp Bot 66: 3215-3227.
653 Parrotta L, Guerriero G, Sergeant K, Cai G, Hausman JF (2015) Target or barrier? The
654 cell wall of early-and later-diverging plants vs cadmium toxicity: differences in the
655 response mechanisms. Front Plant Sci 6: 133.
656 Perilli P., Mitchell LG, Grant CA, Pisante M (2010) Cadmium concentration in durum
657 wheat grain (*Triticum turgidum*) as influenced by nitrogen rate, seeding date and soil
658 type. J Sci Food Agr 90(5): 813-822.
659 Peng JS, Wang YJ, Ding G, Ma HL, Zhang YJ, Gong JM (2017) A pivotal role of cell
660 wall in cadmium accumulation in the Crassulaceae hyperaccumulator *Sedum*

661 *plumbizincicola*. Mol Plant 10: 771-774.
662 Ricachenevsky FK, Menguer PK, Sperotto RA, Williams LE, Fett JP (2013) Roles of
663 plant metal tolerance proteins (MTP) in metal storage and potential use in
664 biofortification strategies. Front Plant Sci 4: 144.
665 Rui H, Chen C, Zhang X, Shen Z, Zhang F (2016) Cd-induced oxidative stress and
666 lignification in the roots of two *Vicia sativa* L. varieties with different Cd tolerances.
667 J Hazard Mater 301: 304-313.
668 Sarwar N, Malhi SS, Zia MH., Naeem A, Bibi S, Farid G (2010) Role of mineral
669 nutrition in minimizing cadmium accumulation by plants. J Sci Food Agr 90: 925-
670 937.
671 Schutzendubel A, Schwanz P, Teichmann T, Gross K, Langenfeld–Heyser R, Godbold
672 Rizwan M, Ali S, Ali B, Adrees M, Arshad M et al (2019) Zinc and iron oxide
673 nanoparticles improved the plant growth and reduced the oxidative stress and
674 cadmium concentration in wheat. Chemosphere 214: 269-277.
675 Sharma A, Sainger M, Dwivedi S, Srivastava S, Tripathi RD, Singh RP (2010)
676 Genotypic variation in *Brassica juncea* (L.) Czern. cultivars in growth, nitrate
677 assimilation, antioxidant responses and phytoremediation potential during cadmium
678 stress. *Journal of Environmental Biology* 31: 773.
679 Sharma SS, Dietz KJ (2006) The significance of amino acids and amino acid-derived
680 molecules in plant responses and adaptation to heavy metal stress. J Exp Bot 57: 711-
681 726.
682 Tian SK, Lu LL, Yang XE, Labavitch JM, Huang YY, Brown P (2009) Stem and leaf

683 sequestration of zinc at the cellular level in the hyperaccumulator Sedum alfredii.
684 New Phytol 182: 116-126.
685 Tian SK, Xie RH, Wang HX, Hu Y, Hou DD, Liao XC, Brown PH, Yang XE, Liu XY,
686 Labavitch JM et al (2017) Uptake, sequestration and tolerance of cadmium at cellular
687 levels in the hyperaccumulator plant species *Sedum alfredii*. J Exp Bot 68: 2387-
688 2398.
689 Yang S, Zu Y, Li B, Bi Y, Jia L, He Y, Li Y (2019) Response and intraspecific differences
690 in nitrogen metabolism of alfalfa (*Medicago sativa* L.) under cadmium stress.
691 Chemosphere 220: 69-76.
692 Zhang J, Martinoia E, Lee Y (2018) Vacuolar transporters for cadmium and arsenic in
693 plants and their applications in phytoremediation and crop development. Plant Cell
694 Physiol 59: 1317-1325.
695 Zhang S, Yang C, Chen M, Chen J, Pan Y, Chen Y, Pan YH, Chen YG, Rahman SU,
696 Fan JF, Zhang, Y (2019) Influence of nitrogen availability on Cd accumulation and
697 acclimation strategy of *Populus* leaves under Cd exposure. Ecotox Environ Safe 180:
698 439-448.
699 Zhao H, Li Y, Duan B, Korpelainen H, Li C (2009) Sex-related adaptive responses of
700 *Populus cathayana* to photoperiod transitions. Plant Cell Environ 32(10): 1401-1411.

Table 1. Net photosynthesis rate (A), stomatal conductance (g_s), transpiration (E), chlorophyll a (Chl a), Chl b and Chl (a+b), and carotenoid (Car) in leaves of *Populus cathayana* females and males, as affected by cadmium stress, N deficiency and their combination.

Sex	Treatment	A	g_s	E	Chl a	Chl b	Chl (a+b)	Car
Female	**+N-Cd (Control)**	17.37±0.80c	0.223±0.026c	4.00±0.48d	1.79±0.031a	0.410±0.031a	2.20±0.074a	0.897±0.029a
	-N-Cd	10.11±1.07ef	0.223±0.035c	3.80±0.44d	0.851±0.13c	0.260±0.091c	1.11±0.23c	0.587±0.017cd
	+N+Cd	12.69±1.66d	0.194±0.024c	3.52±0.31d	1.42±0.13b	0.394±0.14ab	1.82±0.26b	0.492±0.093d
	-N+Cd	9.56±0.64f	0.203±0.029c	3.98±0.37d	0.910±0.097c	0.248±0.035c	1.16±0.18c	0.509±0.012d
Male	**+N-Cd (Control)**	20.96±1.40a	0.368±0.083ab	5.31±0.87ab	1.73±0.076a	0.419±0.11a	2.15±0.12a	0.769±0.01ab
	-N-Cd	12.26±0.26d	0.326±0.052b	4.97±0.52bc	1.34±0.048b	0.304±0.50bc	1.65±0.88b	0.694±0.010bc
	+N+Cd	19.01±1.40b	0.409±0.06a	6.07±0.70a	1.71±0.21a	0.400±0.063ab	2.11±0.23a	0.741±0.20b
	-N+Cd	11.49±1.53de	0.231±0.068c	4.10±0.89cd	1.40±0.12b	0.359±0.029ab	1.76±0.15b	0.754±0.13b
P_s		***	***	***	ns	ns	ns	***
P_{cd}		***	ns	*	**	ns	**	**
P_n		***	**	***	***	***	***	***
$P_{s\times cd}$		ns	ns	ns	ns	ns	ns	***
$P_{s\times n}$		***	**	ns	*	ns	ns	ns
$P_{cd\times n}$		**	ns	***	**	ns	*	**
$P_{s\times cd\times n}$		ns	ns	ns	nsns	ns	ns	ns

F_s, sex effect; P_{cd}, Cd effect; P_n, N effect; $P_{s\times cd}$, the interaction effect of sex and Cd. $P_{s\times n}$, the interaction effect of sex and N; $P_{cd\times n}$, the interaction effect of Cd and N; $F_{s\times cd\times n}$, the interaction effect of sex, Cd and N. Different letters on the bars indicate significant differences between the treatments ($P<0.05$, Duncan's test). The significance values of the three-way analysis of variance are shown as follows: ns, not significant; * $0.01 < P \leq 0.05$; ** $0.001 < P \leq 0.01$; *** $P \leq 0.001$.

Table 2. The dry mass of leaves, stems, roots and total biomass, and the root: shoot ratio in *Populus cathayana* females and males, as affected cadmium stress, N deficiency and their combination.

Sex	Treatment	Leaf mass (g)	Stem mass (g)	Root mass (g)	Total mass (g)	Root : Shoot
Female	**+N-Cd (Control)**	17.01 ± 0.93a	16.76 ± 0.77b	8.33 ± 0.31a	42.10 ± 0.87a	0.247 ± 0.014c
	-N-Cd	7.14 ± 0.84c	4.66 ± 1.06de	3.52 ± 0.42e	16.29 ± 1.62d	0.385 ± 0.074b
	+N+Cd	11.20 ± 1.54b	13.95 ± 1.04c	6.11 ± 0.58b	31.26 ± 1.99b	0.243 ± 0.022c
	-N+Cd	4.04 ± 0.94d	3.83 ± 0.67f	4.49 ± 0.63cd	11.39 ± 1.45e	0.510 ± 0.087a
Male	**+N-Cd (Control)**	8.11 ± 0.24c	18.13 ± 1.32a	4.81 ± 0.83c	31.05 ± 1.52b	0.174 ± 0.019c
	-N-Cd	2.90 ± 1.01de	5.74 ± 0.93d	2.37 ± 0.093f	11.41 ± 0.67e	0.265 ± 0.03c
	+N+Cd	7.03± 0.84c	14.88 ± 0.45c	3.96 ± 0.083de	25.86 ± 0.76c	0.181 ± 0.0086c
	-N+Cd	2.06 ± 0.26d	3.58 ± 0.66f	2.34 ± 0.37f	7.98 ± 0.34f	0.424 ± 0.11b
P_s		***	*	***	***	*
P_{cd}		***	***	***	***	**
P_n		***	***	***	***	***
$P_{s\times cd}$		***	ns	**	***	ns
$P_{s\times n}$		***	ns	**	***	ns
$P_{cd\times n}$		*	*	**	***	ns
$P_{cd\times m\times n}$		ns	ns	ns	*	ns

P_s, sex effect; P_{cd}, Cd effect; P_n, N effect; $P_{s\times cd}$, the interaction effect of sex and Cd. $P_{s\times n}$, the interaction effect of sex and N; $P_{cd\times n}$, the interaction effect of Cd and N; $F_{s\times cd\times n}$, the interaction effect of sex, Cd and N. Different letters on the bars indicate significant differences between the treatments ($P<0.05$, Duncan's test). Values are expressed as means ± SE (n = 6). The significance values of the three-way analysis of variance are shown as follows: ns, not significant; * $0.01 < P \leq 0.05$; ** $0.001 < P \leq 0.01$; *** $P \leq 0.001$.

Figure legends

Figure 1 The concentrations of malondialdehyde (MDA), hydrogen peroxide (H_2O_2) and superoxide radicals (O_2^-) in the leaves and roots of *Populus cathayana* females and males, as affected by cadmium stress, N deficiency and their combination. P_s, sex effect; P_{cd}, Cd effect; P_n, N effect; $P_{s\times cd}$, the interaction effect of sex and Cd. $P_{s\times n}$, the interaction effect of sex and N; $P_{cd\times n}$, the interaction effect of Cd and N; $F_{s\times cd\times n}$, the interaction effect of sex, Cd and N. Different letters on the bars indicate significant differences between the treatments. Values are expressed as means ± SD (n = 4). The significance values of the three-way analysis of variance are shown as follows: ns, not significant; * $0.01 < P \leq 0.05$; ** $0.001 < P \leq 0.01$; *** $P \leq 0.001$.

Figure 2 The activities of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and glutathione reductase (GR) in the leaves and roots of *Populus cathayana* females and males exposed to cadmium stress, N deficiency and their combination. P_s, sex effect; P_{cd}, Cd effect; P_n, N effect; $P_{s\times cd}$, the interaction effect of sex and Cd. $P_{s\times n}$, the interaction effect of sex and N; $P_{cd\times n}$, the interaction effect of Cd and N; $F_{s\times cd\times n}$, the interaction effect of sex, Cd and N. Different letters on the bars indicate significant differences between the treatments. Values are expressed as means ± SD (n = 4). The significance values of the three-way analysis of variance are shown as follows: ns, not significant; * $0.01 < P \leq 0.05$; ** $0.001 < P \leq 0.01$; *** $P \leq 0.001$.

Figure 3 Cd accumulation in the leaves, roots, wood and bark of *Populus cathayana* females and males exposed to cadmium stress, N deficiency and their combination. P_s, sex effect; P_{cd}, Cd effect; P_n, N effect; $P_{s\times cd}$, the interaction effect of sex and Cd. $P_{s\times n}$, the interaction effect of sex and N; $P_{cd\times n}$, the interaction effect of Cd and N; $F_{s\times cd\times n}$, the interaction effect of sex, Cd and N. Different letters on the bars indicate significant differences between the treatments. Values are expressed as means ± SD (n = 4). The significance values of the three-way analysis of variance are shown as follows: ns, not significant; * $0.01 < P \leq 0.05$; ** $0.001 < P \leq 0.01$; *** $P \leq 0.001$.

Figure 4 Cd distribution in the leaf vein cross-sections of *Populus cathayana* females and males exposed to cadmium stress, N deficiency and their combination as determined by energy-dispersive x-ray analysis and scanning electron microscope imaging.

Figure 5 Cd distribution in the leaf blade cross-sections of *Populus cathayana* females and males exposed to cadmium stress, N deficiency and their combination as determined by energy-dispersive x-ray analysis and scanning electron microscope imaging.

Figure 6 Cd distribution in the root cross-sections of *Populus cathayana* females and males exposed to cadmium stress, N deficiency and their combination as determined by energy-dispersive x-ray analysis and scanning electron microscope imaging.

Figure 7 Cd distributions in the root, leaf blade and vein cross-sections of *Populus cathayana* females and males exposed to cadmium stress, N deficiency and their combination as determined by energy-dispersive x-ray analysis and scanning electron microscope imaging.

Figure 8 FTIR spectra and the corresponding principle component analysis (PCA) plot of leaves and roots of *Populus cathayana* females and males exposed to cadmium stress, N deficiency and their combination. The average spectrum of leaves and roots was plotted ($n = 4$). PCA was conducted with the data of selected peaks separately for leaves and roots (Supplementary Table 1).

Figure 9 Principal component analysis (PCA) plots of oxidants, antioxidants, photosynthesis parameters, pigments and biomass in the leaves and roots of *Populus cathayana* females and males exposed to cadmium stress, N deficiency and their combination. PCA was performed using the data presented in Tables 1-2 and Figs 1-2.

Figure 10 Effects of cadmium, nitrogen deficiency and their combination on the expression of *heavy metal ATPase 2 and 4* (*HMA2* and *HMA4*), *metallothionein-like protein* (*MTP1*), *yellow stripe-like protein* (*YSL2*), *zinc transporter 2 and 6.2* (*ZIP2* and *ZIP6.2*) genes in the roots of *Populus cathayana* females and males. Values are expressed as means ± SD (n = 4). Different letters on the bars indicate significant

differences between the treatments (P<0.05, Duncan's test).

Figure 11 A schematic model for Cd accumulation, distribution and tolerance in *Populus cathayana* females and males. CW, cell wall; PM, plasma membrane; ZIP2 and ZIP6.2, zinc/iron regulated transporter 2 and 6.2; ABCCs, ATP-binding cassette transporter ; HMA2, P-type heavy metal ATPase 2; YSL2, yellow stripe-like2.

Figure 1

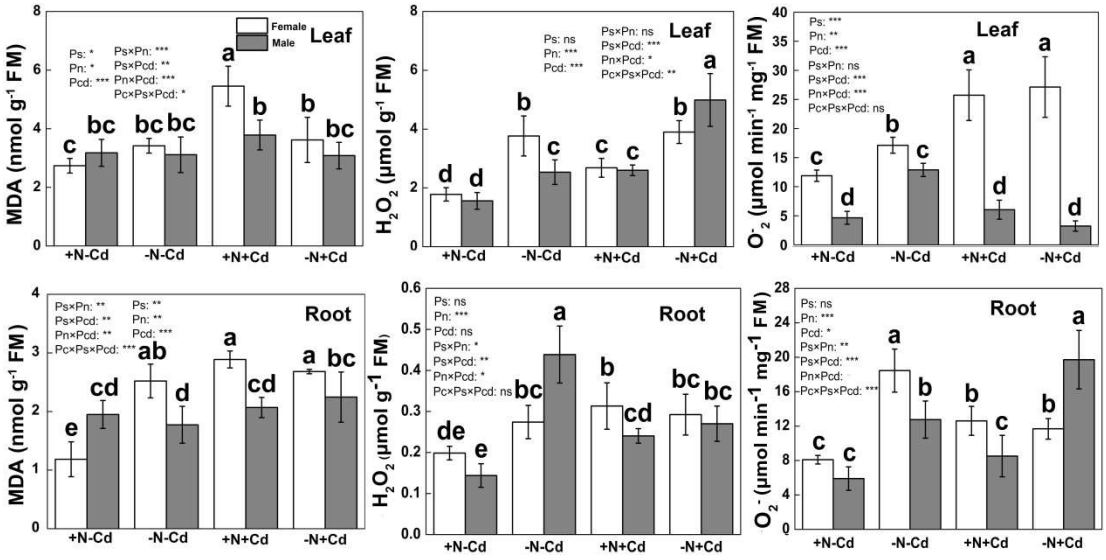

Female
Male
Leaf
Root
MDA (nmol g-1 FM)
H2O2 (μmol g-1 FM)
O2- (μmol min-1 mg-1 FM)
Ps: *
Pn: *
Pcd: ***
Ps×Pn: ***
Ps×Pcd: **
Pn×Pcd: ***
Pc×Ps×Pcd: *
Ps: ns
Pn: ***
Pcd: ***
Ps×Pn: ns
Ps×Pcd: ***
Pn×Pcd: *
Pc×Ps×Pcd: **
Ps: ***
Pn: **
Pcd: ***
Ps×Pn: ns
Ps×Pcd: ***
Pn×Pcd: ***
Pc×Ps×Pcd: ns
Ps×Pn: **
Ps×Pcd: **
Pn×Pcd: **
Pc×Ps×Pcd: ***
Ps: **
Pn: **
Pcd: ***
Ps: ns
Pn: ***
Pcd: ns
Ps×Pn: *
Ps×Pcd: **
Pn×Pcd: *
Pc×Ps×Pcd: ns
Ps: ns
Pn: ***
Pcd: *
Ps×Pn: **
Ps×Pcd: ***
Pn×Pcd:
Pc×Ps×Pcd: ***
+N-Cd
-N-Cd
+N+Cd
-N+Cd

Figure 2

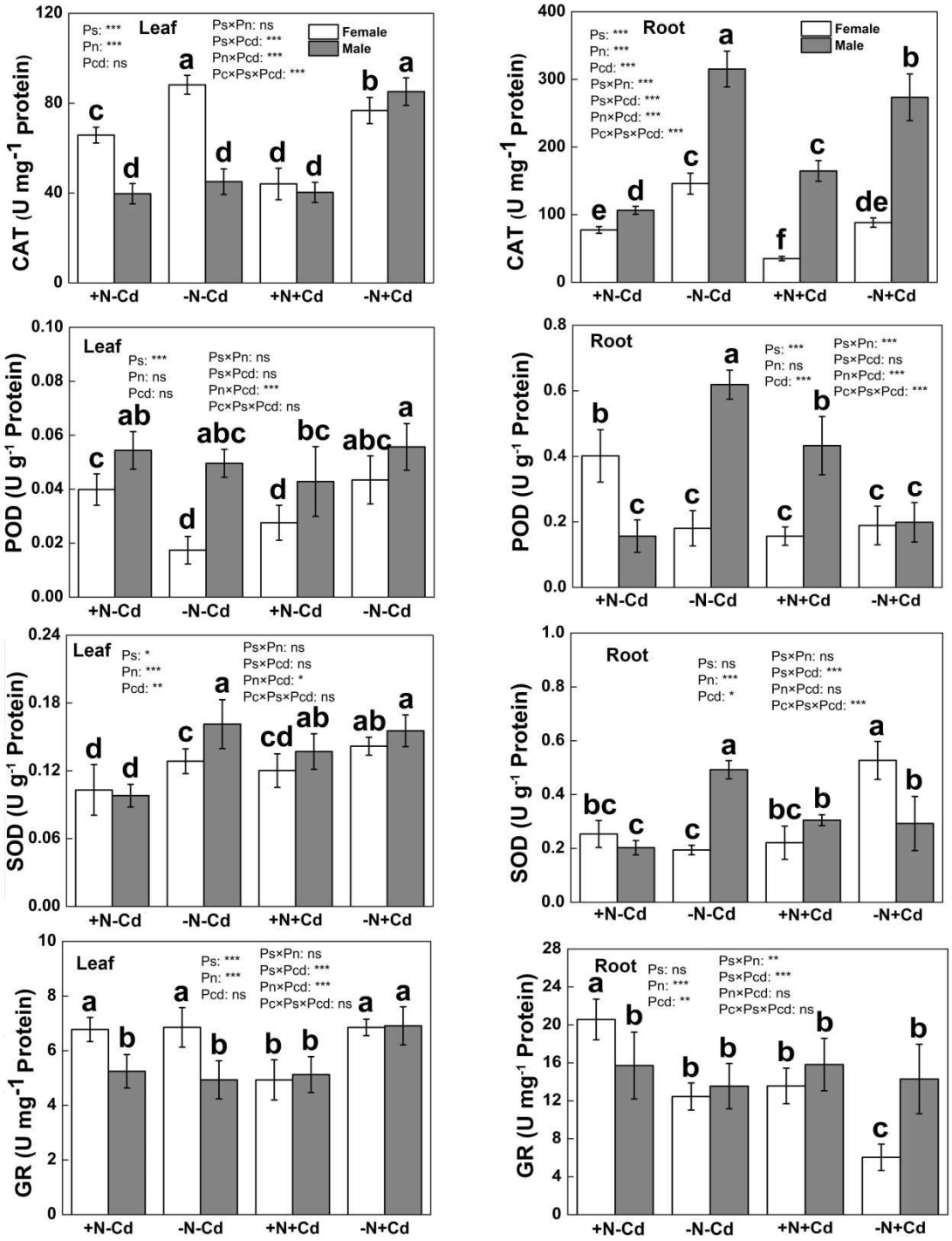

Leaf
Root
Female
Male
CAT (U mg-1 protein)
POD (U g-1 Protein)
SOD (U g-1 Protein)
GR (U mg-1 Protein)
Ps: *** Pn: *** Pcd: ns
Ps×Pn: ns Ps×Pcd: *** Pn×Pcd: *** Pc×Ps×Pcd: ***
Ps: *** Pn: *** Pcd: ***
Ps×Pn: *** Ps×Pcd: *** Pn×Pcd: *** Pc×Ps×Pcd: ***
Ps: *** Pn: ns Pcd: ns
Ps×Pn: ns Ps×Pcd: ns Pn×Pcd: *** Pc×Ps×Pcd: ns
Ps: *** Pn: ns Pcd: ***
Ps×Pn: *** Ps×Pcd: ns Pn×Pcd: *** Pc×Ps×Pcd: ***
Ps: * Pn: *** Pcd: **
Ps×Pn: ns Ps×Pcd: ns Pn×Pcd: * Pc×Ps×Pcd: ns
Ps: ns Pn: *** Pcd: *
Ps×Pn: ns Ps×Pcd: *** Pn×Pcd: ns Pc×Ps×Pcd: ***
Ps: *** Pn: *** Pcd: ns
Ps×Pn: ns Ps×Pcd: *** Pn×Pcd: *** Pc×Ps×Pcd: ns
Ps: ns Pn: *** Pcd: **
Ps×Pn: ** Ps×Pcd: *** Pn×Pcd: ns Pc×Ps×Pcd: ns
+N-Cd
-N-Cd
+N+Cd
-N+Cd

Figure 3

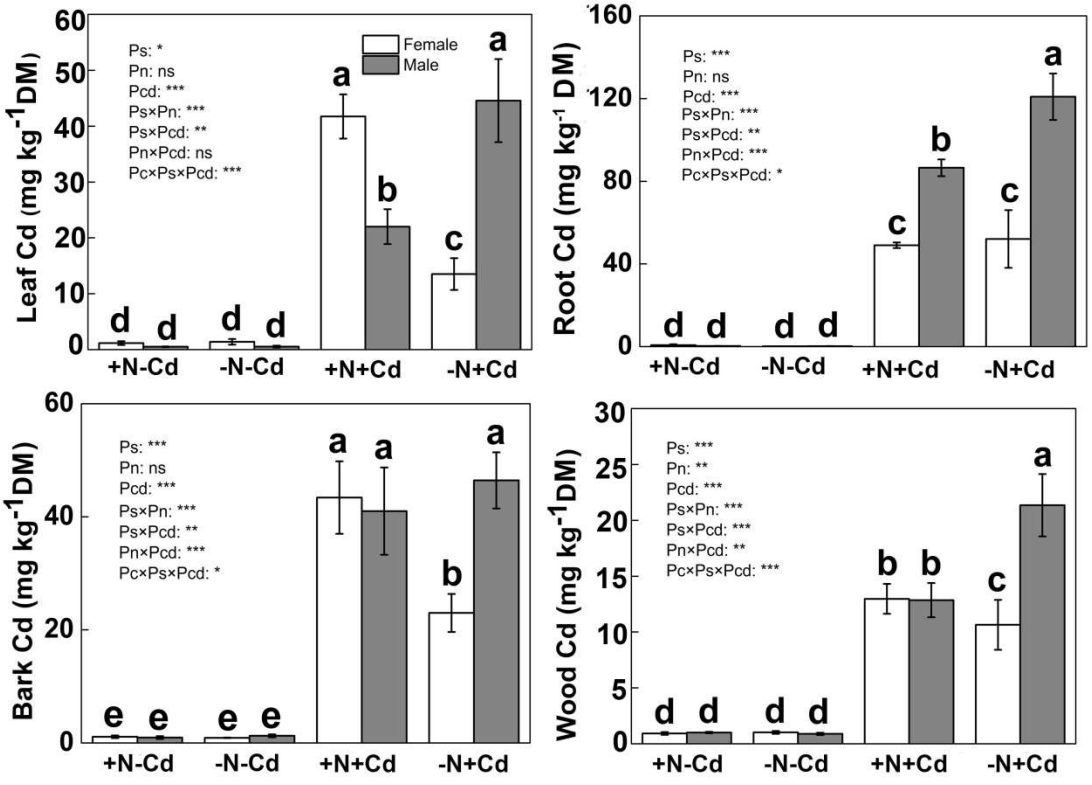

Leaf Cd (mg kg-1DM)
Ps: *
Pn: ns
Pcd: ***
Ps×Pn: ***
Ps×Pcd: **
Pn×Pcd: ns
Pc×Ps×Pcd: ***
Female
Male
+N-Cd
-N-Cd
+N+Cd
-N+Cd
Root Cd (mg kg-1 DM)
Ps: ***
Pn: ns
Pcd: ***
Ps×Pn: ***
Ps×Pcd: **
Pn×Pcd: ***
Pc×Ps×Pcd: *
Bark Cd (mg kg-1DM)
Ps: ***
Pn: ns
Pcd: ***
Ps×Pn: ***
Ps×Pcd: **
Pn×Pcd: ***
Pc×Ps×Pcd: *
Wood Cd (mg kg-1DM)
Ps: ***
Pn: **
Pcd: ***
Ps×Pn: ***
Ps×Pcd: ***
Pn×Pcd: **
Pc×Ps×Pcd: ***

Figure 4

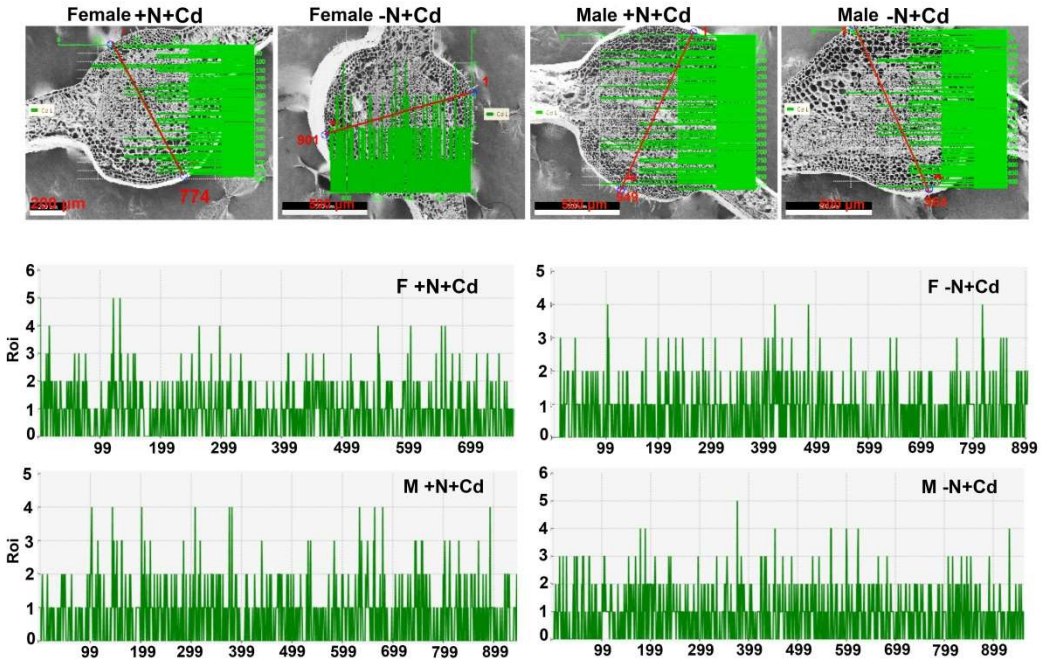

Female +N+Cd
Female -N+Cd
Male +N+Cd
Male -N+Cd
F +N+Cd
F -N+Cd
M +N+Cd
M -N+Cd
Roi
99
199
299
399
499
599
699
799
899

Figure 5

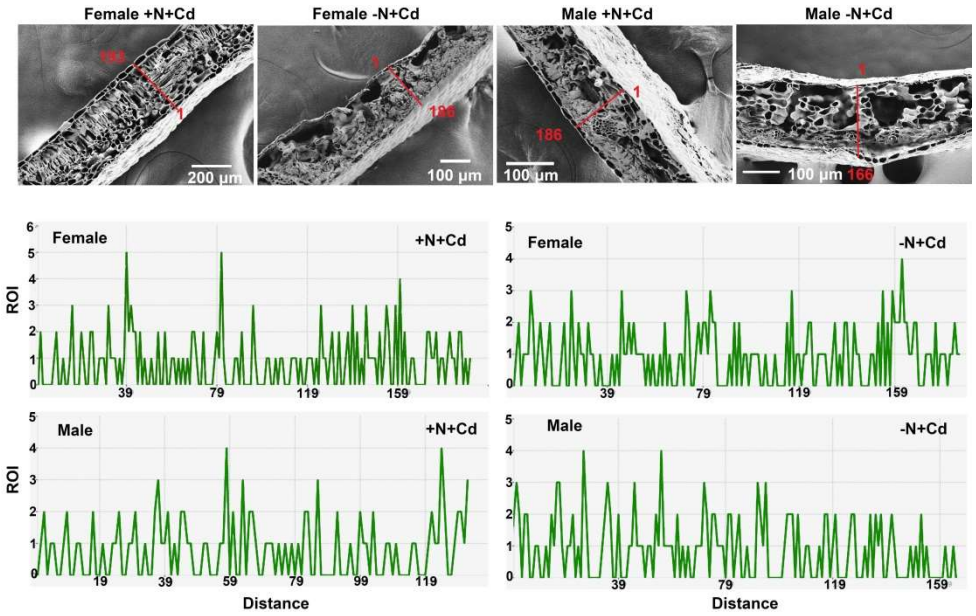

Female +N+Cd
Female -N+Cd
Male +N+Cd
Male -N+Cd
200 µm
100 µm
100 µm
100 µm
Female
+N+Cd
Female
-N+Cd
Male
+N+Cd
Male
-N+Cd
ROI
ROI
Distance
Distance

Figure 6

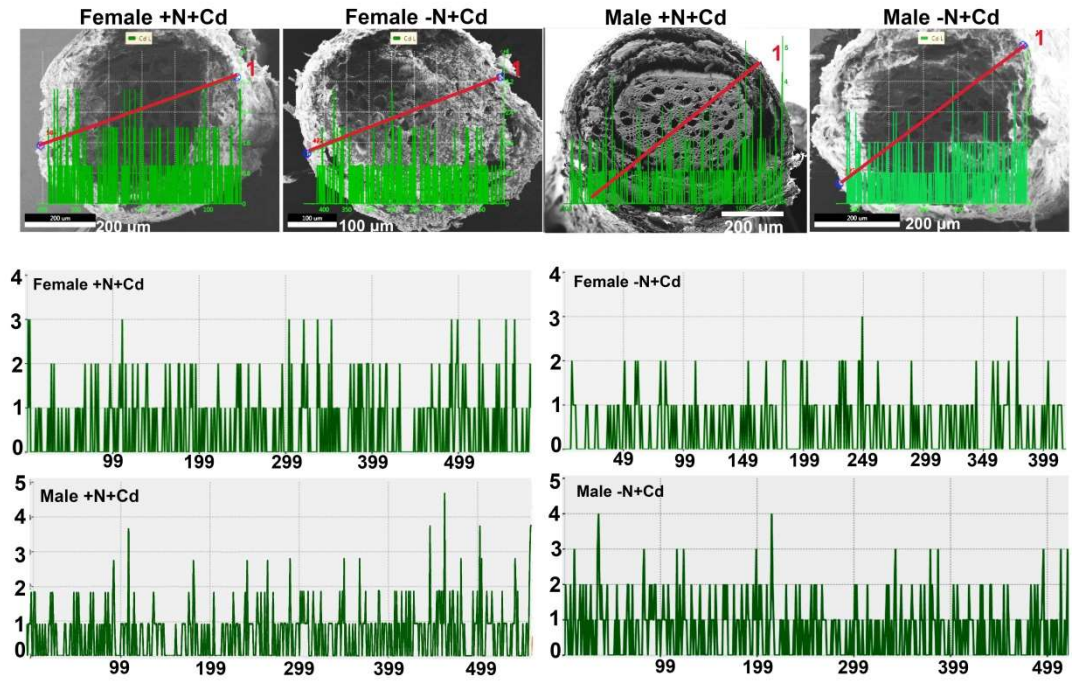

Female +N+Cd
Female -N+Cd
Male +N+Cd
Male -N+Cd
200 µm
100 µm
200 µm
200 µm
Female +N+Cd
Female -N+Cd
Male +N+Cd
Male -N+Cd

Figure 7

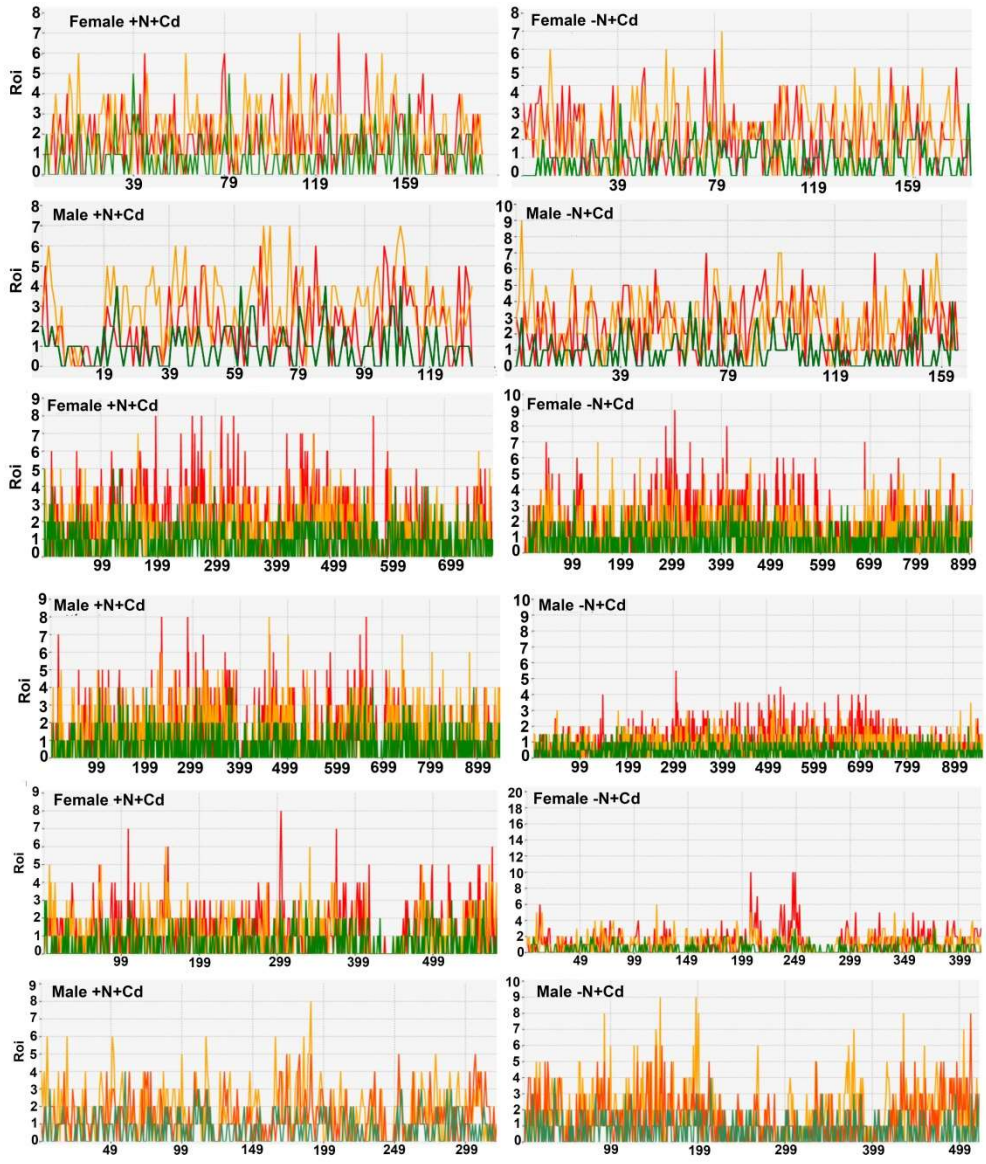

Female +N+Cd
Female -N+Cd
Male +N+Cd
Male -N+Cd
Female +N+Cd
Female -N+Cd
Male +N+Cd
Male -N+Cd
Female +N+Cd
Female -N+Cd
Male +N+Cd
Male -N+Cd
Roi

Figure 8

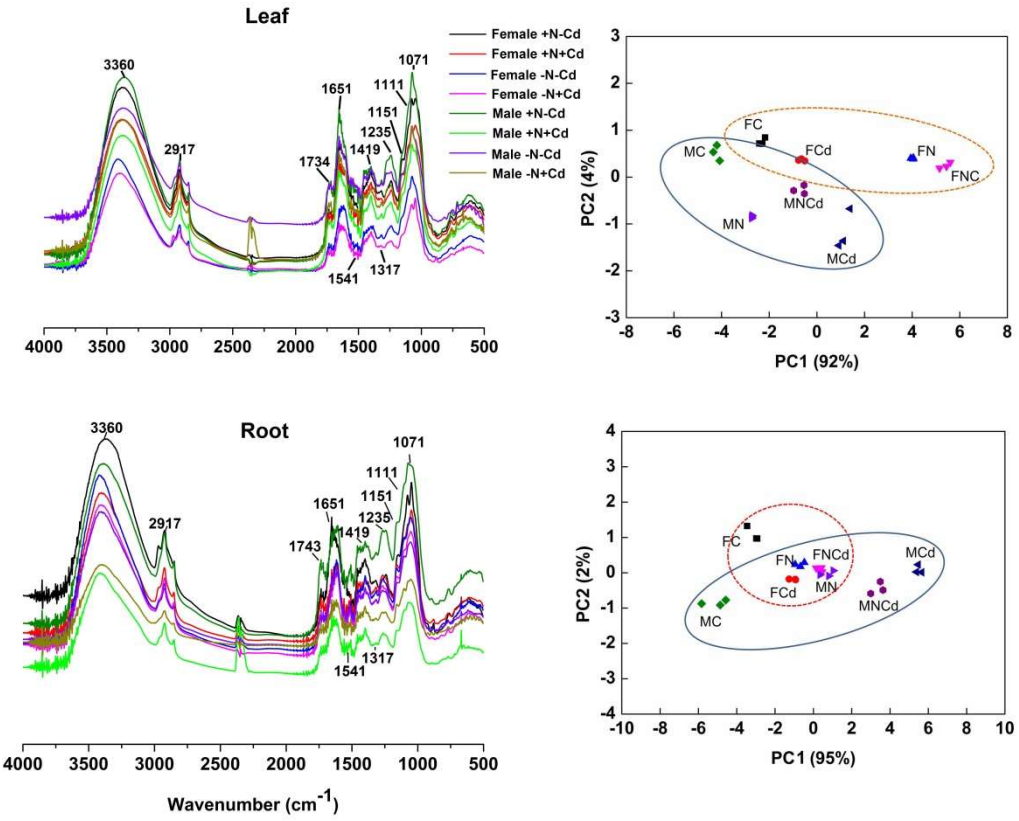

Leaf
Female +N-Cd
Female +N+Cd
Female -N-Cd
Female -N+Cd
Male +N-Cd
Male +N+Cd
Male -N-Cd
Male -N+Cd
3360
2917
1734
1651
1541
1419
1317
1235
1151
1111
1071
4000 3500 3000 2500 2000 1500 1000 500
FC
FCd
FN
FNC
MC
MNCd
MN
MCd
PC2 (4%)
PC1 (92%)
Root
3360
2917
1743
1651
1541
1419
1317
1235
1151
1111
1071
4000 3500 3000 2500 2000 1500 1000 500
Wavenumber (cm-1)
FC
FN
FNCd
FCd
MN
MC
MNCd
MCd
PC2 (2%)
PC1 (95%)

Figure 9

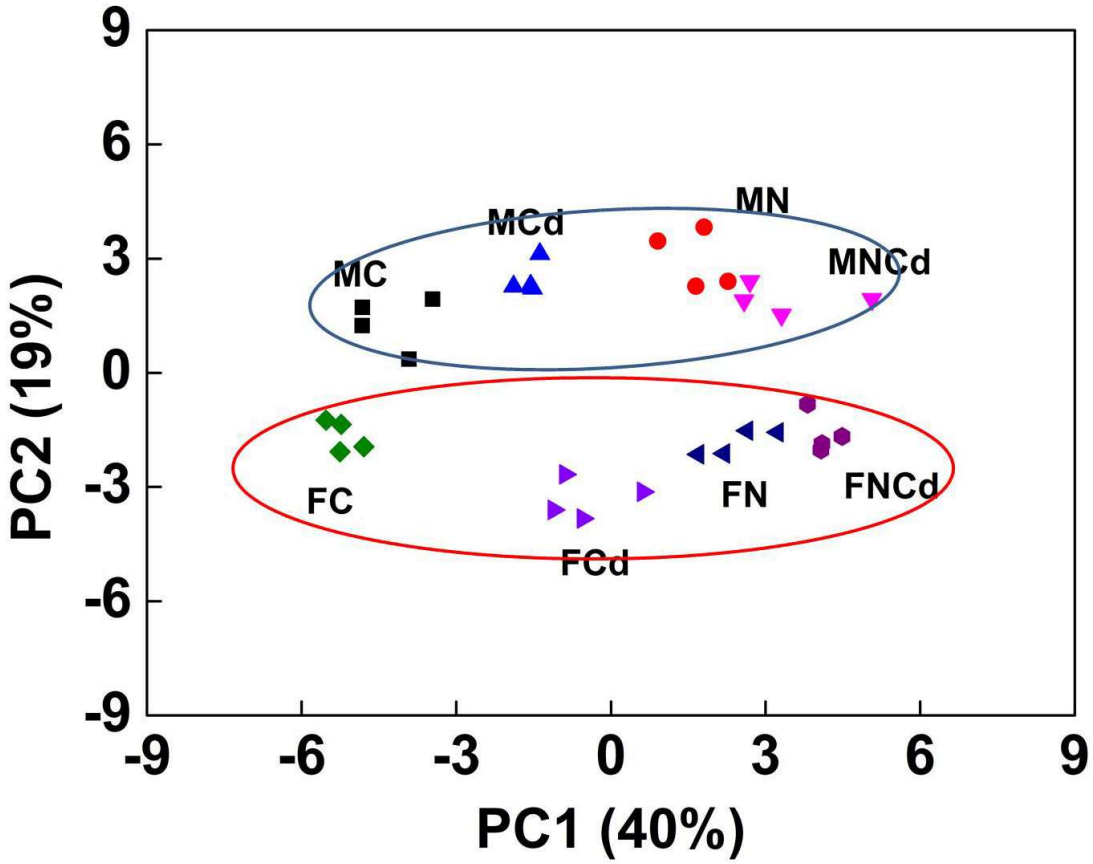


Figure 10

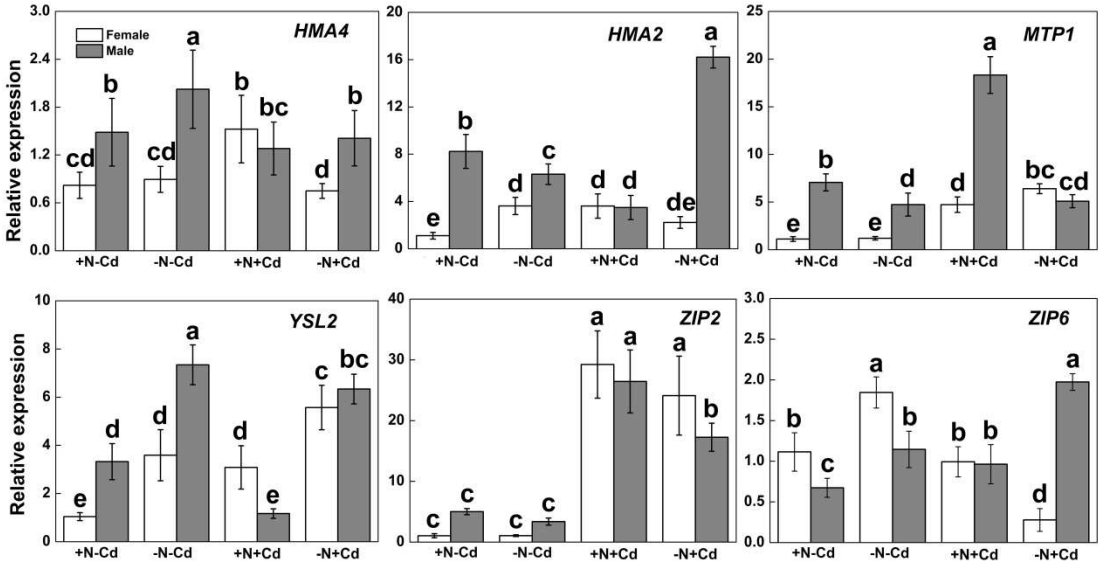
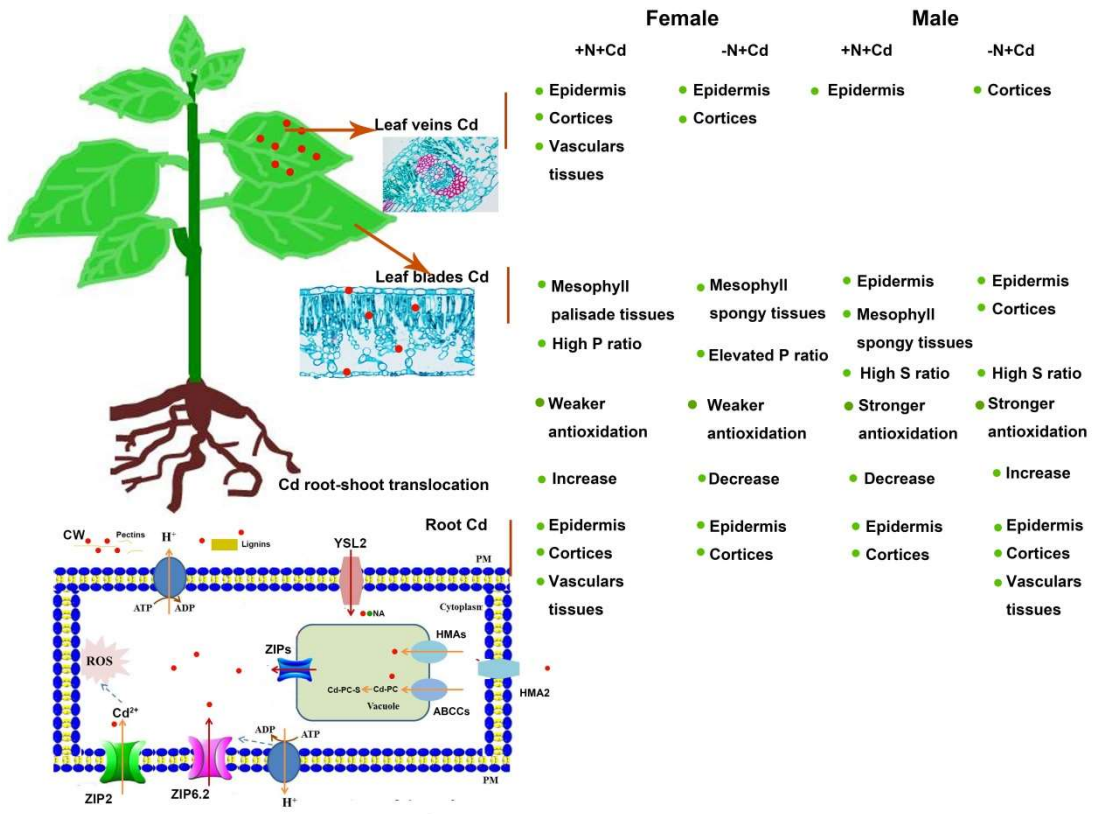

HMA4
HMA2
MTP1
YSL2
ZIP2
ZIP6
Female
Male
Relative expression
+N-Cd
-N-Cd
+N+Cd
-N+Cd

Figure 11


Female
Male
+N+Cd
-N+Cd
+N+Cd
-N+Cd
Leaf veins Cd
Epidermis
Cortices
Vasculars tissues
Epidermis
Cortices
Epidermis
Cortices
Leaf blades Cd
Mesophyll palisade tissues
High P ratio
Mesophyll spongy tissues
Elevated P ratio
Epidermis
Mesophyll spongy tissues
High S ratio
Epidermis
Cortices
High S ratio
Cd root-shoot translocation
Weaker antioxidation
Weaker antioxidation
Stronger antioxidation
Stronger antioxidation
Increase
Decrease
Decrease
Increase
Root Cd
Epidermis
Cortices
Vasculars tissues
Epidermis
Cortices
Epidermis
Cortices
Epidermis
Cortices
Vasculars tissues
CW
Pectins
H+
Lignins
YSL2
PM
ATP
ADP
NA
Cytoplasm
HMAs
ZIPs
ROS
Cd-PC-S
Cd-PC
Vacuole
ABCCs
HMA2
Cd2+
ADP
ATP
ZIP2
ZIP6.2
H+
PM